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Role of environmental factors for the vertical distribution (0–1000 m) of marine bacterial communities in the NW Mediterranean Sea

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Abstract

Bacterioplankton play a central role in energy and matter fluxes at the sea, yet the factors that constrain their variation in the marine systems are still poorly understood. Here we show the explanatory power of multivariate statistical analysis of bacterial community structures coupled with fine measurements of numerous environmental parameters. We gathered and analysed data from a one month sampling period from the surface to 1000 m depth at the JGOFS-DYFAMED station (NW Mediterranean Sea). This station is characterized by very poor horizontal advection currents what makes it an ideal model to test hypothesis on the causes of vertical stratification of bacterial communities. Capillary electrophoresis single strand conformation polymorphism (CE-SSCP) fingerprinting profiles analyzed using multivariate statistical methods demonstrated a vertical zonation of bacterial assemblages in three layers, above, in or just below the chlorophyll maximum and deeper, that remained stable during the entire sampling period. Through the use of direct gradient multivariate ordination analyses we demonstrate that a complex array of biogeochemical parameters is the driving force behinds bacterial community structure shifts in the water column. Physico-chemical parameters such as phosphate, nitrate, salinity and to a lesser extend temperature, oxygen, dissolve organic carbon and photosynthetically active radiation acted in synergy to explain bacterial assemblages changes with depth. Analysis of lipid biomarkers of the organic matter sources and fates suggested that bacterial community structure at the surface layers was in part explained by lipids from chloroplast origin. Further detailed analysis of pigment-based phytoplankton diversity gave evidence of a compartmentalized influence of several phytoplankton groups on bacterial community structure in the first 150 m depth. This study is probably the first example of an analysis employing a complex environmental dataset in combination with microbial community profiles to unravel the mechanisms underneath bacterial assemblages in marine systems.

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1 Introduction

Microorganisms represent the most abundant, biogeochemically important biological component in the oceans. At concentrations ranging between 10^4 and 10^6 cells ml^{-1}, an estimated 20 to 50% of marine primary productivity is channeled through bacterioplankton (Azam et al., 1983; Cho and Azam, 1988, 1990). Bacterial species composition is an important variable controlling the rates and patterns of organic matter remineralization (Martinez et al., 1996; Pinhassi et al., 1997). Thus, knowledge of the composition of bacterial communities, and how that composition varies over space and time, is likely to be of major importance for understanding the role of bacteria in marine biogeochemistry (Riemann et al., 1999). The application of molecular biology techniques to microbial ecology has led to an increased appreciation of bacterial community changes with depth as demonstrated in a wide variety of oligotrophic seawaters, including the Pacific and Atlantic Oceans (Lee and Fuhrman, 1991), the Mediterranean Sea (Acinas et al., 1997; Moeseneder et al., 2001; Ghiglione et al., 2005, 2007), the Arabian Sea (Riemann et al., 1999) and Antarctic zones (Murray et al., 1998). The presence of the same pattern in such widely distant ocean basins suggests that vertical stratification is a very important factor determining the bacterial species inhabiting the ocean (Acinas et al., 1997). Previous studies formulated different hypotheses to explain the vertical distribution of bacterial communities pointing to by depth-related changes in environmental conditions or interactions with other planktonic components. Thus, the existing uncertainty underlines the need for a more robust investigation of this question. More generally, the factors that constrain microbial community composition and its variation in marine systems are still poorly understood in marine systems (Polz et al., 2006). We hypothesized that the combination of high-throughput diversity composition assessments in combination with in-depth environmental parameters measurements and appropriate multivariate statistical analyses should shed light on the actual factors responsible for bacterioplankton communities shifts with depth.

The NW Mediterranean Sea is characterized by oligotrophic conditions and strong

isothermic stratification of the water column during the summer. The great depth (2300 m), and weakness of horizontal advection currents (Andersen and Prieur, 2000), makes the French JGOFS-DYFAMED sampling area situated at the central zone of the Ligurian Sea (NW Mediterranean) a great model zone to follow the mineralization fluxes from the prokaryotic compartment along the water column. In this work, we examined the vertical distribution (0–1000 m) of bacterial community structure at this station during a one month cruise, by using capillary electrophoresis-single strand conformation polymorphism (CE-SSCP). This technique, originally developed for mutation detection, has been widely used for genetic profiling of microbial communities within a variety of ecosystems (Lee et al., 1996; Sunnucks et al., 2000; Godon et al., 2001; Ghiglione et al., 2005) and presents similar or higher detection limits than DGGE or T-RFLP fingerprinting techniques (Hong et al., 2007; Smalla et al., 2007). CE-SSCP high reproducibility permits comparisons of microbial community fingerprinting profiles from a large set of samples (Ghiglione et al., 2005). As a first objective we sought to determine the consistency of the vertical distribution of bacterial communities during the late summer–autumn transition period by using multivariate non-parametric statistical methods. Secondly, we took quantitatively unparallel measurements of environmental parameters including physico-chemical variables, lipid class biotracers and phytoplankton pigments, to investigate their influence as potential driving forces for shifts in bacterial community structure along the water column using multivariate statistical direct gradient ordination methods. In a companion paper, the vertical and temporal dynamics of bacterial abundance and activity during this cruise are reported in Mével et al. (2008).

2 Materials and methods

2.1 Study site and general sampling methods

Sampling took part during a one month cruise conducted from mid-September to mid-October 2004 at the French-JGOFS time-series station DYFAMED (43°25′2 N, 07°51′8 E; 2350 m max depth) in the NW Mediterranean Sea on the RV "Thalassa". We collected samples once a week on 19 September (Julian Day – JD 263; CTD 46) (sample A), 26 September (JD 270; CTD 101) (sample B), 6 October (JD 280; CTD 167) (sample C) and 12 October (JD 286, CTD 215) (sample D) using a rosette system equipped with 24 Niskin bottles. For each sampling day, samples were collected at depths between the surface and 1000 m (i.e. 5, 20, 40, 60, 80, 150, 200, 400, 500, 700, 1000 m).

2.2 Physico-chemical parameters

We used standard sensors of the CTD – SeaBird SBE 911 plus installed in the rosette system to measure pressure, temperature, conductivity, oxygen, salinity and photosynthetically active radiation (PAR).

For nutrients (nitrate, nitrite, phosphate, silicate) seawater was collected into polyethylene flasks and analyses were performed on board on a Technicon AutoAnalyzer (AAII) according to Tréguer and LeCorre (1975). Ammonia was measured on board according to Holmes et al. (1999) on a Turner Design TD-700 fluorimeter.

For dissolved organic carbon (DOC), seawater was filtered through two precombusted (24 h, 450°C) glass fiber filters (Whatman GF/F, 25 mm) and collected into precombusted glass tubes closed with a screw cap and a Teflon liner. Samples were acidified with orthophosphoric acid (H_3PO_4) and DOC was analyzed immediately on board on a Shimadzu TOC-V analyser using the high temperature catalytic oxidation (HTCO) technique (Sugimura and Suzuki, 1988; Cauwet, 1994). Analytical accuracy of measurements was less than 2 μM. Deep Sargasso Sea reference

water (47 mol l^{-1} C, ±0.5 SE, http://www.rsmas.miami.edu/groups/biogeochem/CRM.html) was injected every 10 samples to insure stable operating conditions.

Colored dissolved organic matter (CDOM) substances were extracted from 500 ml sea water samples by adsorption on C18 microcolumns. CDOM was eluated from the microcolumn by methanol and quantified by light-absorbance at 412 nm.

2.3 Lipid biomarkers

Four liters of seawater were filtered onto pre-combusted glass fiber filters (Whatman GF/F, 47 mm). Dissolved lipids were extracted from 2 l of the filtrate by liquid-liquid extraction, using dichloromethane (two extractions at sea water natural pH, followed by two extractions at pH 2). Each lipid extract was separated into classes of compounds according to polarity, using chromarods SIII (0.9 mm diameter, 150 mm length, 75 μm silica thick) and quantified using a thin layer chromatography-flame ionization detection (TLC-FID) Iatroscan TH10 apparatus model MK-IV (Iatron, Japan; hydrogen flow, 160 ml min^{-1}; air flow, 2000 ml min^{-1}). The elution scheme previously described (Striby et al., 1999) separated twelve classes of acyl-lipids and free lipids, including reserves (wax esters (WE) and triacylglycerols (TG)), lipids from chloroplasts (LC) (pigments and glycolipids), cellular membrane lipids (phospholipids (PL) which include phosphoglycerides, phosphatidylethanolamines and phosphatidylcholines), and their hydrolysis metabolites (Met) (alcohols, free fatty acids, monoglycerides and 1,2- and 1,3-diglycerides).

2.4 Phytoplankton pigments

Two liters and a half of seawater were filtered on glass fiber filters (Whatman GF/F, 25 mm), which were immediately frozen in liquid nitrogen until analysis. Pigments analysis employed a Agilent Technologies HPLC system with a RP-C8 (Agilent) column 150 mm long, 3 mm internal diameter, 3.5 μm granulometry (Zorbax Eclipse) and a Diode array detector (1100 model). All steps of extraction and analysis were per-

formed under dim light conditions. The use of an internal standard (Vitamin E Acetate) allowed for the correction of possible losses during extraction as well as for the presence of water in the filter. The analytical method was based on a separation gradient between a methanol: ammonium acetate (70:30 v:v) mixture and 100% methanol as in Vidussi et al. (1996) with a few improvements in sensitivity.

Identification of pigments was performed using retention-time data and by comparison of on-line collected absorption spectra with those of a library established from standards and reference cultures. Response factors for eight external pigment standards (peridinin, 19′-butanoyloxyfucoxanthin, fucoxanthin, 19′-hexanoyloxyfucoxanthin, alloxanthin, zeaxanthin, chlorophyll-*b*, chlorophyll-*a*) were determined by spectrophotometry (Perkin Elmer) followed by HPLC analysis. These response factors were then derived to compute the specific extinction coefficients of these pigments for the HPLC system. The specific extinction coefficients from others pigments were derived from the literature (Jeffrey et al., 1997). The concentration of key chlorophyll and carotenoid pigments was used to determine phytoplankton community composition.

A review on taxonomic pigments can be found in Jeffrey and Vesk (1997). Divinyl chlorophyll-*a* and Divinyl chlorophyll-*b* are typical markers of picoplanktonic prochlorophytes (Goericke and Repeta, 1992; Claustre et al., 1994), and zeaxanthin is contained in both prochlorophytes and synechoccocus. 19′ hexanoyloxyfucoxanthin is a marker of prymnesiophytes (Wright and Jeffrey, 1987) and 19′butanoyloxyfucoxanthin is mainly associated with pelagophytes (Andersen et al., 1993) but also with prymnesiophytes (Jeffrey et al., 1997). In the microphytoplankton range fucoxanthin is associated with diatoms (Barlow et al., 1997) but is also present in prymnesiophytes and pelagophytes. Peridinin is a specific marker of autotrophic dinoflagellates (Jeffrey and Vesk, 1997). The CHEMTAX matrix factorization program developed by Mackey et al. (1998) was applied to the phytoplankton data set to determine the contribution of principal taxonomic phytoplankton groups to the total chlorophyll-*a* (Marty et al., 2008[1]).

[1]Marty J. C., Garcia, N., and Raimbault, P.: Phytoplankton dynamics and primary production under late summer conditions (DYNAPROC II cruise Sep/Oct 2004, NW Mediterranean Sea),

2.5 Nucleic acid extraction and PCR of 16S rDNA

Two liters of seawater were pre-filtered through 3 μm-pore-size filters (47 mm, Nucleopore) to remove most eukaryotic organisms and to prevent clogging of the final filter. Bacterial cells were concentrated onto 0.22 μm-pore-size filters (47 mm, PC Nucleopore) and stored into 2 ml Eppendorf tubes at −20°C until extraction. The procedure used for DNA extraction was modified from Furhman et al. (1988). This is a relatively gentle method that employs enzymatic and detergent-based lysis to avoid excessive shearing of DNA, producing DNA of suitable quality for PCR amplification, and reducing the risk of chimera formation during PCR. Previously frozen filters were cut with sterilized scissors into small strips and vortexed briefly in 840 μl of alkaline lysis buffer (50 mM Tris hydrochloride pH=8.3, 40 mM EDTA, 0.75 M sucrose). Cells were lysed by an initial incubation for 45 min at 37°C after adding 50 μl of freshly prepared lysosyme solution (20 mg ml^{-1}), and a second incubation at 50°C for 10 min after adding 100 μl of 10% sodium dodecyl sulfate and 10 μl of a proteinase K solution (20 mg ml^{-1}). The lysate was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and an equal volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated at −20°C overnight with 1 volume of isopropanol, and resuspended in TE (10 mM Tris, 1 mM EDTA). Molecular weight distribution and purity of the DNA were assessed by 1% agarose gel electrophoresis and quantified either by visual comparison with molecular weight markers in ethidium bromide stained agarose gels (rough estimate) or by optical density measurements using GeneQuant II (Pharmacia Biotech.).

PCR amplification was performed by using primers w49 (5′-ACG GTC CAG ACT CCT ACG GG-3′) and w34 (5′-TTA CCG CGG CTG CTG GCA C-3′), which amplify the variable V3 region of the 16S rDNA (Escherichia coli positions 329–533) (Brosius et al., 1981). The primer w34 was fluorescently labelled at the 5′-end with phosphoramidite (TET, Applied Biosystems). Both primers were synthesized commercially (Eurogentec). Each 50 μl reaction mixture contained 50 μM of each primer, 1X Pfu reaction

buffer, 20 mM dNTPs, 1.0 U of Pfu DNA polymerase (Promega) and 0.1 μg of template DNA. PCR amplification consisted of an initial denaturation step of 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min using a Robocycler (Stratagene). Correct size (ca. 200 bp length) of PCR products were determined by 2% agarose gel electrophoresis with a DNA size standard (Low DNA Mass Ladder, GIBCO BRL).

2.6 Capillary Electrophoresis – Single Strand Conformation Polymorphism (CE-SSCP) procedure

Labelled PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen) according to manufacturers instructions followed by quantification by visual inspection as above. Samples were diluted in sterile TE (10 mM Tris, 1 mM EDTA) in order to obtain 10 ng μl^{-1} of PCR product. From the resulting dilution, 1 μl of PCR product was mixed with 18 μl of formamide (Applera) and 1 μl of an internal size standard GeneScan-400 Rox (Applied Biosystems). The mixture was then denatured for 5 min at 94°C and immediately cooled on ice for at least 5 min. The procedure used for CE-SSCP analysis was a modification of that described by Delbès et al. (1998). Samples were electrokinetically injected (5 s, 12 kV) in a capillary tube (47 cm×50 μm) filled with a mixture composed of 5.6% GeneScan polymer (Applied Biosystems) and 10% autoclaved glycerol in sterile TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]). Electrophoresis was carried out at 15 kV and 30°C for 30 min per sample and phosphoramidite (TET)-labelled amplicons were detected by a laser with a virtual filter C (detection wavelengths 532, 537, and 584 nm). Data were collected with ABI Prism 310 collection software (Applied Biosystems). In order to normalize mobilities from different runs, all electropherograms were calibrated by fixing the positions of peaks produced by the size standard and by using a second-order least square curve (i.e. linear regression) to provide the best interlane comparison (Genescan analysis software, Applied Biosystems). Electropherograms were then transferred to the software SAFUM (Zemb

et al., 2007), which renders a profile of fluorescence intensity as a function of retention time per sample taking therefore into account the presence and intensity of each individual signal. This software corrects for sample intensity differences rendering normalized profiles for all samples.

2.7 Ecological statistical analysis

Comparative analysis of CE-SSCP fingerprints was carried out with the PRIMER 5 software (PRIMER-E, Ltd., UK). Ordination of Bray-Curtis similarities among normalized sample profiles was performed by non-metric multidimensional scaling (MDS). We used this ordination technique to determine the relationships among sample profiles as representative of the bacterial community structure of each sample site. This method attempts to preserve the ranked order of the similarity of any two communities as an inverse function of the distance between plotted sample points (Kruskal, 1964). The degree to which the plot matches the similarity matrix can be judged by examining stress values (Kruskal's stress). MDS was performed using 30 random starting configurations of sample points to avoid local minima. Additionally, hierarchical agglomerative clustering of Bray-Curtis similarities was performed using the group average method of PRIMER software. To test the null hypothesis that there was no difference between bacterial communities of different depth layers we conducted an analysis of similarities with the subroutine ANOSIM of PRIMER. ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA) (Clarke and Warwick, 2001). ANOSIM first calculates the R statistic that displays the degree of separation between groups. Complete separation is indicated by R=1, whereas R=0 suggests no separation. Having determined R, ANOSIM randomly assigns samples to different groups to generate a null distribution of R (Monte Carlo test) to test whether within-group samples are more closely related to each other than would be expected by chance.

To investigate relationships between bacterioplankton community structure and physico-chemical parameters, lipids biomarkers and phytoplankton pigments, we used

a direct gradient approach, canonical correspondence analysis (CCA) using the software package CANOCO, version 4.5 for Windows (ter Braak and Šmilauer, 2002). CCA generates an ordination plot that shows the main pattern of variation in community composition as accounted for by measured environmental variables. In other words, the resulting ordination axes are linear combinations of the environmental variables that best explain microbial diversity composition data (ter Braak, 1986). We first imported OTUs abundance data from spreadsheets using WCanoImp program within the CANOCO package. We then used Canoco program to perform CCA with species scaling on intersample distances so that samples and environmental variables formed a biplot. To statistically evaluate the significance of the first canonical axis and of all canonical axes together we used Monte Carlo permutation full model test with 199 unrestricted permutations. Finally, to represent biplots we used the program CANODRAW within CANOCO package. Additionally, Spearman's rank pairwise correlations between the environmental variables mentioned above helped to determine their significance for further ecological analysis.

3 Results

3.1 Bacterial community structure analysis

Bacterial community structure was characterized through CE-SSCP fingerprinting profiling once per week within 0–1000 m depth during September–October 2004. An example of the community fingerprints obtained at different depths for julian day 263 is shown in Fig. 1. On a mean of 49 peaks (SD=0.82, n=4) found at each date, 19.4% (±5.23) were found at all depth whereas 14.9% (±0.82) appeared only once or twice in the water column. For comparative analysis of community fingerprints we used multivariate MDS and hierarchical agglomerative clustering. Both analyses show a recurrent in-depth pattern along the sampling period with distinct bacterial communities at three different layers: 0–40 m, 60–150 m and 200–1000 m (Fig. 2). MDS ordination

plot stress value was low (0.14) which indicates that the ordinations unlikely to mislead interpretation (Clarke and Warwick, 2001). We then used ANOSIM to determine the significant differences among bacterial communities from the three depth layers. This analysis showed the highest difference between surface and deeper community structures (R=0.754, p=0.001). Bacterial communities from the intermediate layer were closer to the upper layer than to the deeper layer but still significantly different from both (R=0.32 p=0.001 and R=0.22, p=0.004, respectively).

3.2 Bacterial community structure and physico-chemical variables ecological analysis

We first performed CCA using all physico-chemical parameters as constrained variables of the bacterial community diversity from CE-SSCP fingerprints of 0–1000 m depth samples. CCA revealed values higher than 20 of the variance inflation factor for nitrate and silicate, which indicated that these variables were almost perfectly correlated with other variables in the dataset. A strong Spearman's rank pairwise correlation between nitrate and silicate (R=0.91, p<0.01) allowed us to use nitrate as a proxy of silicate to perform CCA together with the rest of physico-chemical parameters. A summary of the results of this CCA is shown in Table 1. The total variance of fitted 0–1000 m samples diversity data when using physico-chemical variables to explain their bacterial community structure was 0.237, as indicated by the sum of all canonical eigenvalues (ter Braak and Šmilauer, 2002). The cumulative percentage variance of species-environment relation indicates that the first and second canonical axes account for 38.4 and 17.9% of this variance respectively (Table 1). Consequent axes accounted for less than 10% of the variance each, and are not further considered here. The eigenvalues of the ordination analyses, which also measure their relative importance are small (<0.3) implying that CCA does not strongly separates species niches and therefore the biplot rule must be used to interpret the fitted relative abundances of samples with respect to physico-chemical variables in the CCA ordination diagram (Fig. 3). Monte Carlo test for first and all canonical axes were highly significant (p<0.01) indicating that the parameters selected are good explanatory variables of

the community structure. First canonical axis is highly negatively correlated with depth, salinity, nitrate and PO_4 (ca. −0.5) and positively correlated with oxygen and DOC (ca. 0.4) and to a lesser extend with temperature and PAR (ca. 0.2). This first synthetic gradient clearly separates samples in the three depth layers observed when using diversity data alone indicating that their community composition varies with variation in concentrations of these parameters. Because depth was not collinear with the other variables correlated with first axis, we include this variable in the analysis as a proxy of pressure effect on bacterial community structure. Surface water samples further spread out along the second canonical axis (Fig. 3). We infer that 60 m depth samples have similar concentrations of nitrite, ammonia and CDOM variables elements than 40 m samples and together form a group independent from that of the 5–20 m samples (with the exception of C-20m sample). The significant contribution of these variables in defining surface bacterial community structures was confirmed by additional CCAs using only shallower samples (data not shown).

3.3 Bacterial community structure and lipid biomarkers ecological analysis

CCA total variance of the fitted CE-SSCP diversity from 0–1000 m depth samples when using dissolve lipid biomarkers to explain this diversity is 0.093 with the first two axes accounting for 72.5% of this variance (Table 1). Eigenvalues for these axes were 0.050 and 0.018, respectively. The Monte Carlo test for first and all canonical axes rendered a significant p-value of 0.02. The lipid biomarkers explain therefore the difference in bacterial community structures among samples according to the CCA biplot in Fig. 4. The first synthetic gradient was strongly positively correlated with lipids from chloroplast origin (LC, ca. 0.8) followed by triglycerides (TG, ca. 0.4). Samples from the upper 0–20 m layer, and especially samples from time B-5 m and 20 m, time C-5 m and 20 m and time D-20 m were separated from the rest according to this first axis. The rest of samples formed a relatively tighter group with no clear difference between layers at this first axis indicating the homogeneity of LC at these samples (Fig. 4). Some samples from this last deeper group further spread out along the second axis, which is slightly

positively correlated with TG (ca. 0.3), and to a lesser extent with the remaining lipid biomarkers analyzed in this study. However, samples appear mixed with respect to their depth along this axis.

3.4 Distribution of phytoplankton pigments

Because phytoplankton pigments were almost undetectable below 150 m depth, all analyses with phytoplankton diversity data included 0–150 m depth samples only. Total chlorophyll-*a* (TChl-*a*) maxima increased during the one month cruise period of this study from 0.45–0.44 $\mu g\,l^{-1}$ at 50 m depth for times A and B, respectively, to 0.60 $\mu g\,l^{-1}$ at 40 m depth for time C and 0.80 $\mu g\,l^{-1}$ at 50 m depth for time D. The study period was characterized by the predominance of small species (nano- and pico-phytoplankton) over microphytoplankton. The principal classes observed were prochlorophytes, cyanobacteria (*Synechococcus*) together with prymnesiophytes, pelagophytes and prasinophytes (with concentrations of these classes varying from 15 to 19% of TChl-*a*). Diatoms and dinoflagellates were lesser constituents of the phytoplankton (from 3 to 5% of TChl-*a*). We observed very important variations in the vertical distribution of specific biomasses. As an example, euglenophytes were minor constituents of the TChl-*a* biomass (<2%) and present at very low concentrations during the whole sampling period except for their high concentration (almost 10% of TChl-*a*) in the surface layer 0–20 m at date A (Marty et al., 2008[1]).

3.5 Bacterial community structure and phytoplankton pigments ecological analysis

CCA when using pigment analysis-based phytoplankton diversity to explain CE-SSCP bacterial community structure gave a total variance of fitted 0–150 m samples diversity data of 0.200 (Table 1). Axes 1 and 2 accounted for 56.2% of this variance being the other axes negligible. Eigenvalues for the two first axes were 0.064 and 0.048 respectively. Monte Carlo permutation tests of first and all canonical axes were highly significant (p-values≤0.01). Canonical axis 1 is highly positively correlated with *Syne-*

chococcus and euglenophytes (ca. ∼0.5) and, to a lesser extend, positively correlated with dinoflagelates and negatively to pelagophytes and diatoms (ca. ±0.3) (Fig. 5). This first axis clearly distinguished between samples from the two shallower depth layers described above based on higher concentrations of *Synechococcus*, euglenophytes and dinoflagelates in the 0–20 m depth samples vs. the higher levels of pelagophytes and diatoms in the deeper 60–150 m layer. The second axis was correlated with higher abundances of prasinophytes and prochlorophytes further distinguishing the bacterial communities from samples at 40–60 m with the exception of B-60 m and C-60 m samples. Primnesiophytes, chlorophytes and cryptophytes variation had slight influence on the distribution of samples as indicated by their small arrow length (Fig. 5).

4 Discussion

4.1 CE-SSCP incomes to bacterial community profiling studies

Culture-independent, sequence-based community fingerprinting techniques such as CE-SSCP (Lee et al., 1996), terminal restriction fragment length polymorphism (T-RFLP) (Marsh, 1999), or automated ribosomal intergenic spacer analysis (A-RISA) (Borneman and Triplett, 1997) allow comparison of a theoretically infinite number of samples. Application of these techniques to environmental samples yields considerable amount of information on their microbial diversity composition that can be used to assess how environmental factors contribute to changes in microbial community structure. However, the introduction of experimental biases during analysis and the interpretation of the complex data generated still represent major challenges for the application of these methods in microbial ecology. Biases during nucleic acid extraction, PCR amplification (Wintzingerode et al., 1997) and 16S rDNA copy number variability (Farrelly et al., 1995) may lead to a distorted view of bacterial community structure. In this study, several tests were performed to investigate the efficiency and reproducibility of the DNA extraction and PCR–CE–SSCP protocols. Cell lysis was monitored several

times by microscopy, which assured that there was no cells recalcitrant to our lysis procedure. The quality of the DNA was carefully assessed before proceeding to 16S rDNA gene amplification. Only single PCR products were used for subsequent analyses. The use of an internal standard co-migrating with each sample PCR product allowed us to normalize peak mobilities for comparative sample analysis. This procedure results in a high reproducibility of both, peak pattern and peak intensities, as previously observed (Ghiglione et al., 2005). Finally, because all samples in this study are subject to similar biases, comparative analysis of microbial community structure and relative abundances are still possible (Hughes et al., 2001). On the other hand, the use of adequate numerical and statistical tools overcomes the difficulty of analyzing complex datasets. Until now most studies have used indirect gradient analysis such as principal component analysis or cluster analysis to infer the influence of environmental variables on microbial diversity data, leaving the use of direct gradient analysis approaches such as redundancy analysis or canonical correspondence analysis (CCA) neglected (Ramette, 2007). Here we compared both indirect and direct gradient multivariate approaches to explore relationships between bacterial community structure as obtained by CE-SSCP with respect to concomitant measurements of physico-chemical parameters, lipid class biotracers and pigment analysis-based phytoplankton diversity.

4.2 Indirect gradient multivariate analysis to study bacterial community composition

The DYFAMED offshore station is an ideal sampling site for 1-D studies because it is far enough from the Ligurian current to be protected from lateral transport (Andersen and Prieur, 2000). During the late summer-autumn oligotrophic conditions, biotic parameters showed a temporarily stable organization along the 0–1000 m water column. Bacterial community structure appeared differentiated in three layers, shallow (5–40 m), intermediate (60–150 m) and mesopelagic (200–1000 m), when using multivariate (Fig. 2) and cluster analyses. The robustness of this strong stratification with depth pattern was confirmed by a greater similarity within than between samples from each of these layers. This study adds to previous findings of a strong stratification pat-

tern with depth of bacterial communities in oligotrophic conditions (Lee and Fuhrman, 1991; Acinas et al., 1997; Murray et al., 1998; Riemann et al., 1999; Moeseneder et al., 2001; Ghiglione et al., 2007). Vertical and temporal dynamics of bacterial abundance and activity during this cruise are depicted in Mével et al. (2008).

4.3 Linking bacterial community profiling to the environment by direct gradient multivariate analysis

To unravel the environmental parameters eventually responsible for the diversity pattern observed, we chose direct gradient canonical correspondence analysis (CCA). Long ago developed by community ecologists to elucidate the relationships between biological assemblages of species and their environment, it is only recently that CCA has been applied to microbial assemblages from marine (Cordova-Kreylos et al., 2006; Klaus et al., 2007; Sapp et al., 2007), lake (Yannarell and Triplett, 2005) and soil (Salles et al., 2004) systems. Problems with this method may arise when explanatory variables are intercorrelated, making indistinguishable their separate effects. To avoid this problem, we performed separate analysis of measured environmental variables by organizing them in three ranks: physico-chemical variables, lipid biomarkers and phytoplankton pigments. We also observed Spearman's rank pairwise correlations between variables within each rank and avoided co-linear variables when performing CCA.

4.4 CCA with physico-chemical parameters

Our CCA analyses, using physico-chemical data as explanatory variables, showed a temporarily stable vertical stratification of the bacterial community. At the DYFAMED station, under the oligotrophic conditions observed, the diversity data alone is statistically significantly related to changes in concentrations of salinity, nitrate and phosphate variables and to a lesser extend oxygen, DOC, PAR and temperature (Fig. 3). Physical and chemical parameters are usually studied in combination with bacterial activity or bacterial growth to demonstrate their influence on the prokaryotic component of the ma-

rine food web (Caron, 1994; Downing et al., 1999), sometimes with specific changes along the water column (Riemann et al., 1999). At the DYFAMED station, bacterial activity was limited by phosphorus above the deep-chlorophyll maximum (DCM) level (0–40 m) and by carbon at intermediate depth (60–150 m) for the same seasonal sampling time as in our study (Van Wambeke et al., 2002). To our knowledge, however, no studies have demonstrated the direct influence of environmental parameters on the bacterial community structure of natural environmental gradients. The originality of the present study is to demonstrate that several physico-chemical parameters are acting in synergy with nearly equal contribution to the bacterial vertical stratification. A complex interaction of physico-chemical parameters is therefore necessary to predict bacterial community structure.

4.5 CCA with lipid class biotracers

Since lipid classes are useful markers to determine sources and fate of organic matter in marine systems (Parrish, 1988) they have widely been used as biological tracers (Parrish et al., 1995; Derieux et al., 1998; Goutx et al., 2000), but only once in conjunction with bacterial community structure changes (Ghiglione et al., 2007). In the present study CCA analysis showed that dissolved lipids from chloroplast origin (LC), which indicate phytoplankton source of DOM (Goutx et al., 1990), were the main biotracer associated with bacterial community structure, due surely to their high concentration at particular samples of 5–20 m depths (Fig. 4). Inputs of LC from microalgae to the dissolved carbon pool might occur through cell lysis, grazing by herbivorous and/or viral lysis. We found a significant correlation between dissolved LC concentrations and both *Synechoccocus* cells number and *Synechoccocus* to total picophytoplankton ratio (R^2=0.55 and R^2=0.61, p<0.01, n=36, respectively). This result, together with pico-phytoplancton (*Synechococcus* and *Prochlorococcus*) dominance in the sampling site (see Results) let us infer that *Synechoccocus* cells are the major source of LC pools in our samples. The other four classes of dissolved lipid biomarkers contributed to a lesser extent to bacterial community structure differences among samples along

the water column perhaps because their biological signature is more difficult to track. Phospholipids (PL) are membrane compounds in biological cells (Gérin and Goutx, 1993), wax esters (WE), are storage compounds in zooplankton and bacteria (Sargent et al., 1977; Ishige et al., 2003), trigycerides (TG) are reserve compounds in zooplankton, microalgae, bacteria and flagellates (Lee et al., 1971; Parrish and Wangersky, 1987; Alvarez and Steinbüchel, 2002; Méjanelle et al., 2005) and metabolites (Met) are products of acyl-lipid hydrolysis, free fatty acids, free alcohols, diglycerides and monoglycerides (Goutx et al., 2003). Their marginal effect upon the sample scores in the ordination diagram is probably because, taken together, these biomarkers indicated the presence of freshly biosynthesized organic matter not exclusively from phytodetritus.

4.6 CCA with pigment analysis-based phytoplankton diversity

It is well known that marine phytoplankton biomass and species distributions vary along vertical profiles, an obvious example being the deep chlorophyll maximum found in many areas of the world's oceans. This distribution may affect prokaryotic assemblages as well, since phytoplankton distribution appears to be one of the most important biotic variables influencing planktonic bacterial growth (Azam et al., 1983; Cole et al., 1988). Some studies have proposed a possible relation between bacterial community structure and phytoplankton, but most of them were limited to the study of chlorophyll-*a* pigments. Phytoplankton pigment signatures obtained by high-pressure liquid chromatography are widely used as a proxy of phytoplankton diversity in marine systems (Bustillos-Guzman et al., 1995; Mackey et al., 1998; Marty et al., 2002). We used this signature to determine how phytoplankton taxonomic composition explained bacterial community CE-SSCP profiles. We found that the stratification of bacterial community structures in two shallow layers, 0–40 m and 60–150 m, coincided with the higher presence of *Synechococcus*, euglenophytes and dinoflagelates in the first 40 m samples, while diatoms and pelagophytes were preferentially present at samples bellow 60 m. Furthermore, separation of samples situated at the chlorophyll-*a* maximum cor-

responds with a higher abundance of prochlorophytes and prasinophytes at this depth. The general preferential abundance of some of these groups along the water column coincides with our findings, particularly for *Synechococcus* (Veldhuis et al., 2005), diatoms (Latasa et al., 1992), and pelagophytes (Claustre et al., 1994). It is also the case for prochlorophytes which are preferentially located at the base of the chlorophyll maximum (Li and Wood, 1988; Marty et al., 2002) and for prasinophytes which have been described as forming localized maxima at depth in the Mediterranean Sea (Marie et al., 2006). Euglenophytes and dinoflagelates presence at the surface layers is particular to this study. On the other hand, because of their uniform distribution in the first 150 m depth, it is not surprising that prymnesiophytes, cryptophytes and chlorophytes had low influence on bacterial community changes with depth. Our results are the first study to demonstrate a compartmentalized influence of each phytoplankton group on bacterial community structure stratification in the first 150 m of the water column.

5 Concluding remarks

Our study is perhaps the first example of such a complex biogeochemical dataset, which includes physico-chemical parameters, lipid class biotracers and pigment analysis-based phytoplankton diversity, analyzed together with microbial community profiles to unravel the mechanisms responsible for bacterial community structuring with depth in the marine environment. Direct gradient multivariate canonical analyses using these parameters as explanatory environmental variables of the molecular fingerprinting profiles along the water column revealed that the observed vertical zonation of bacterial communities in three layers, above, in or just below the chlorophyll maximum and deeper, is due to synergetic driving forces pertaining to the three biogeochemical levels analyzed in this study. Similarly, the interaction of several of these parameters further distinguished samples situated above and in the chlorophyll maximum (up to 150 m). The role of the quality of the organic matter (DOC and CDOM) is significant in the upper layers (Fig. 3) and we have been able to attribute it to a phytoplankton

origin (Figs. 4 and 5). The influence of phytoplankton was not limited to exudates released at the surface levels since different phytoplankton phylogenetic groups explain the bacterial community structure zonation in the first 150 m depth (Fig. 5). This finding is consistent with a notion of specific association between bacteria and phytoplankton. This study set the basis to further investigations of the plausible mechanisms responsible of bacterioplankton distribution in marine systems. For instance, by enlarging the array of ecological factors explored such as virus or protozoan predation or competition between bacteria for carbon and nutrient sources.

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Table 1. Summary of results from canonical correspondence analyses of the bacterioplankton community structure data when constrained by physico-chemical, lipid biomarkers or phytoplankton environmental variables, respectively.

Environmental variables	Physico-chemical (0–1000 m)		Lipid biomarkers (0–1000 m)		Phytoplankton (0–150 m)	
Total inertia	0.518		0.415		0.449	
Sum of all canonical eigenvalues	0.237		0.093		0.200	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Eigenvalues	0.091	0.042	0.050	0.018	0.064	0.048
Species-environment correlations	0.926	0.876	0.761	0.714	0.959	0.850
Cumulative percentage variance of						
– species data	17.6	25.8	9.6	13.0	14.3	25.1
– species-environment relation	38.4	56.3	53.6	72.5	32.0	56.2

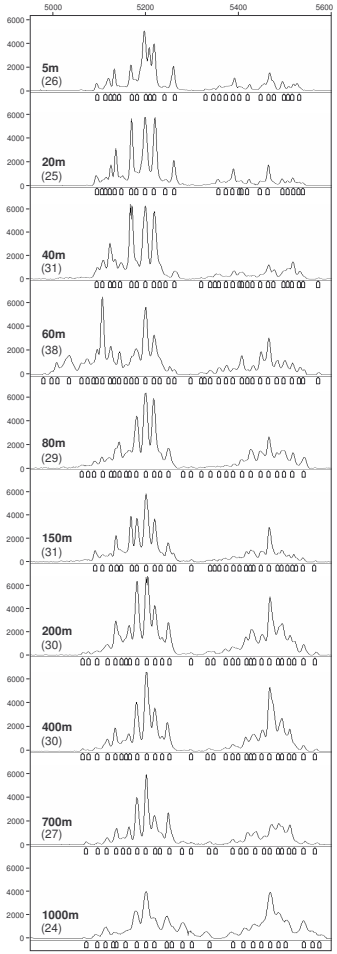

Fig. 1. Depth profiles of CE-SSCP fingerprints of bacterial communities of sample A (Julian Day 263).

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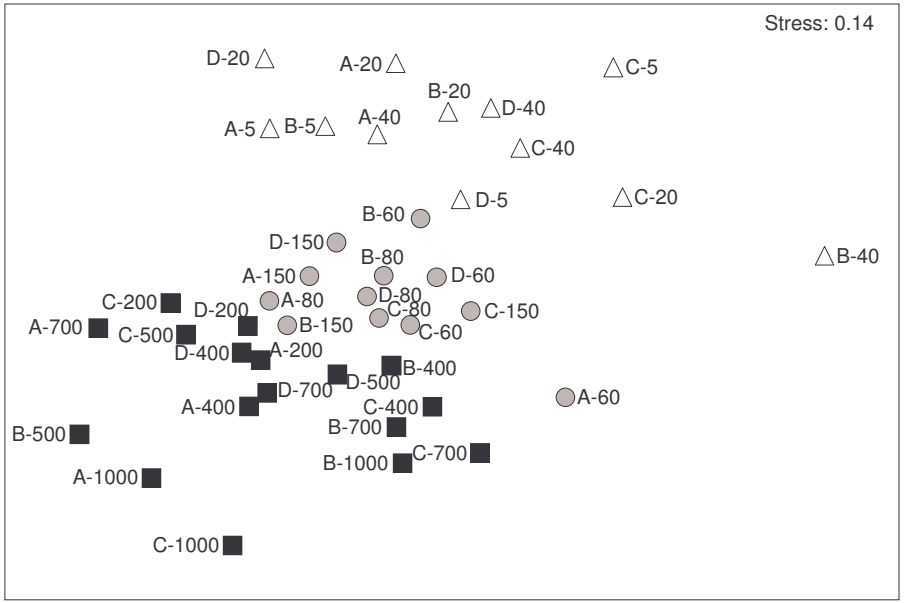

Fig. 2. MDS plot based on Bray-Curtis similarities of CE-SSCP fingerprints of bacterial communities samples at different time and depth. Depth repartition in three groups: 0–40 m (Δ), 60–150 m (◯) and 200–1000 m (■).

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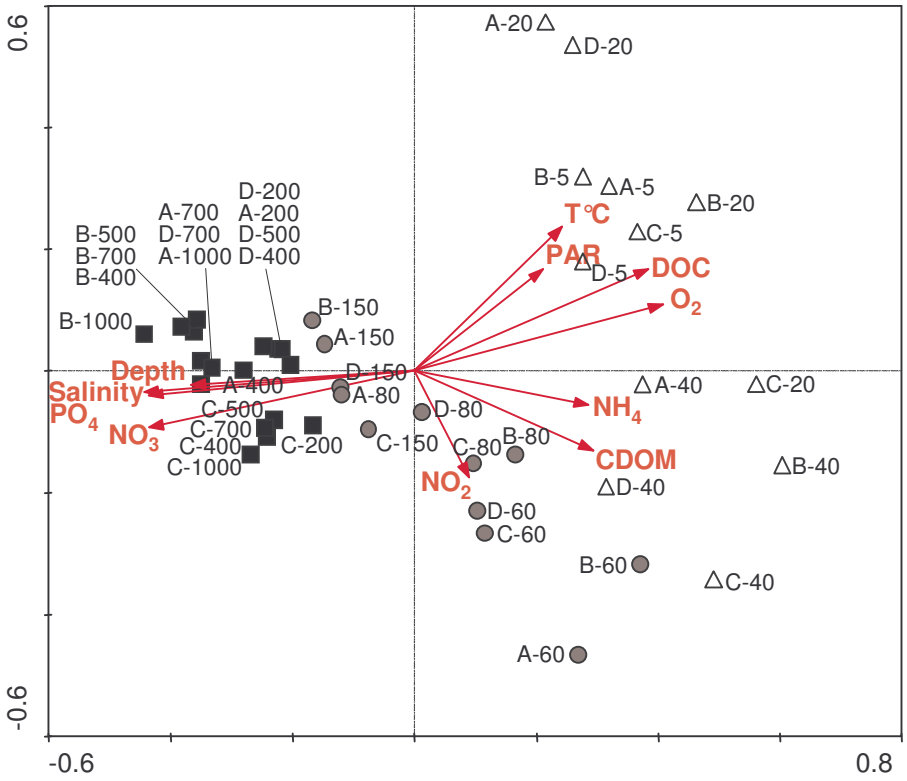

Fig. 3. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 1000 m depth using physico-chemical parameters. Arrows point in the direction of increasing values of each variable. The length of the arrows indicates the degree of correlation with the represented axes. The position of samples relative to arrows is interpreted by projecting the points on the arrow and indicates the extent to which a sample bacterial community composition is influenced by the environmental parameter represented by that arrow. Samples from different depth groups (0–20 m, 60–150 m and 200–1000 m) are indicated by different symbols.

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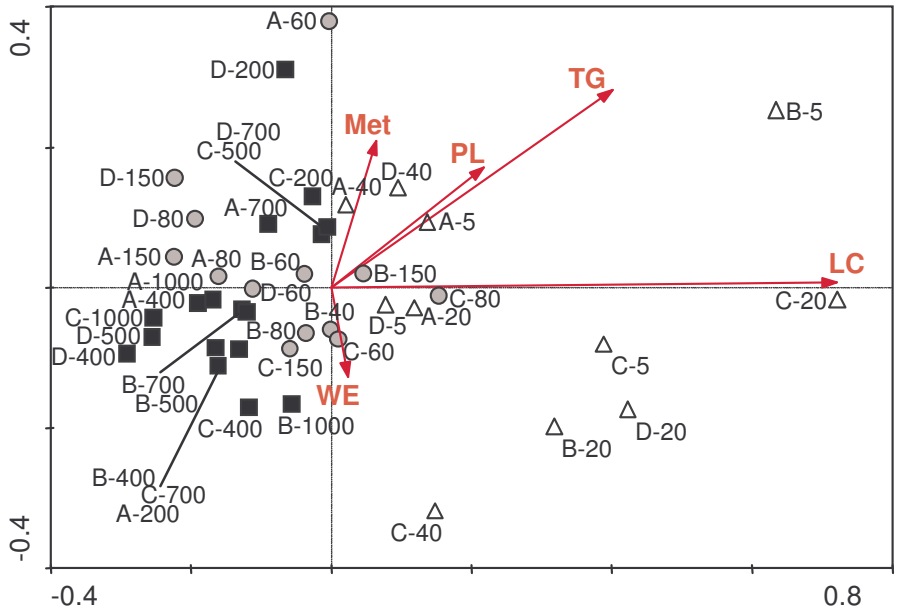

Fig. 4. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 1000 m depth using lipid biomarker parameters. For interpretation see Fig. 3. LC: lipids from chloroplastic origin, TG: triglycerides, Met: metabolites, PL: phospholipids, WE: wax esters.

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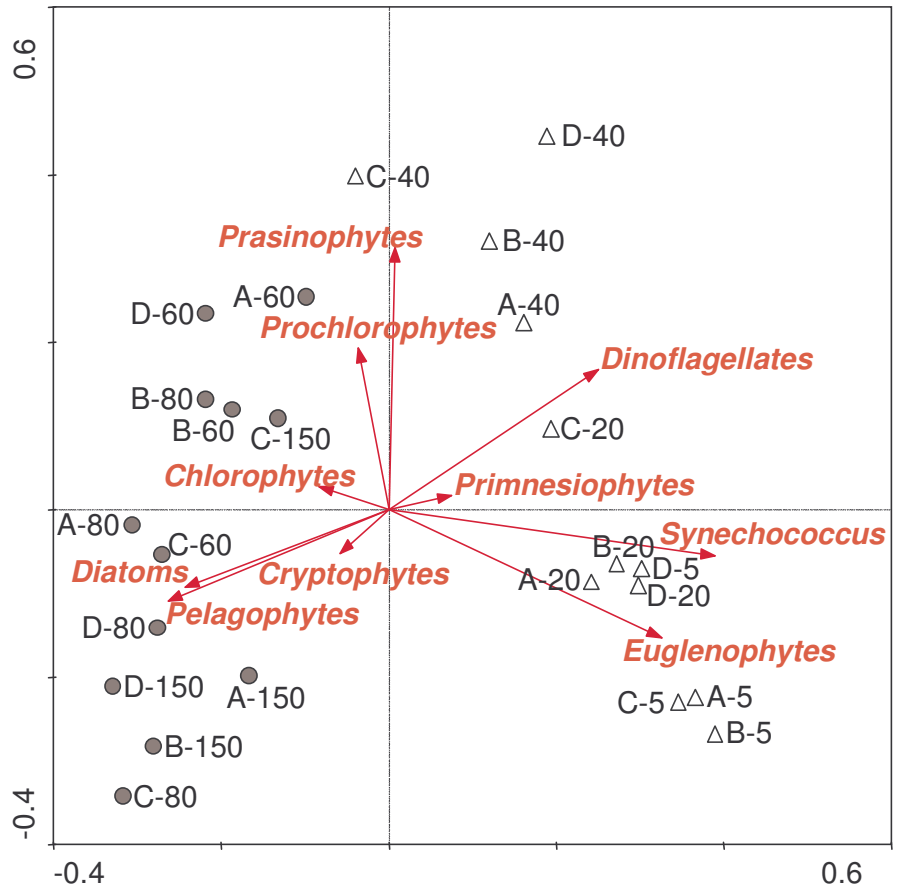

Fig. 5. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 150 m depth using phytoplankton pigments variability. For interpretation see Fig. 3.

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